

Discovery of β -Arrestin–Biased Dopamine D₂ Ligands for Probing Signal Transduction Pathways Essential for Antipsychotic Efficacy

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Elucidating the key signal transduction pathways essential for both antipsychotic efficacy and side-effect profiles is essential for developing safer and more effective therapies. Recent work has highlighted noncanonical modes of dopamine D₂ receptor (D₂R) signaling via β -arrestins as being important for the therapeutic actions of both antipsychotic and antimanic agents. We thus sought to create unique D₂R agonists that display signaling bias via β -arrestin–ergic signaling. Through a robust diversity-oriented modification of the scaffold represented by aripiprazole (1), we discovered UNC9975 (2), UNC0006 (3), and UNC9994 (4) as unprecedented β -arrestin–biased D₂R ligands. These compounds also represent unprecedented β -arrestin–biased ligands for a G_i-coupled G protein–coupled receptor (GPCR). Significantly, UNC9975, UNC0006, and UNC9994 are simultaneously antagonists of G_i-regulated cAMP production and partial agonists for D₂R/ β -arrestin-2 interactions. Importantly, UNC9975 displayed potent antipsychotic-like activity without inducing motoric side effects in inbred C57BL/6 mice in vivo. Genetic deletion of β -arrestin-2 simultaneously attenuated the antipsychotic actions of UNC9975 and transformed it into a typical antipsychotic drug with a high propensity to induce catalepsy. Similarly, the antipsychotic-like activity displayed by UNC9994, an extremely β -arrestin–biased D₂R agonist, in wild-type mice was completely abolished in β -arrestin-2 knockout mice. Taken together, our results suggest that β -arrestin signaling and recruitment can be simultaneously a significant contributor to antipsychotic efficacy and protective against motoric side effects. These functionally selective, β -arrestin–biased D₂R ligands represent valuable chemical probes for further investigations of D₂R signaling in health and disease.

functional selectivity | ligand bias

G protein–coupled receptors (GPCRs) signal not only via canonical pathways involving heterotrimeric large G proteins, but also via noncanonical G protein–independent interactions with other signaling proteins including, most prominently, β -arrestins (1–4). The process by which GPCR ligands differentially modulate canonical and noncanonical signal transduction pathways is a phenomenon known as “functional selectivity” (5, 6). Such functionally selective ligands preferentially engage either canonical or noncanonical GPCR pathways (7, 8). Clearly, the discovery of ligands with discrete functional selectivity profiles will be extremely useful for elucidating the key signal transduction pathways essential for both the therapeutic actions and the side effects of drugs (6). Understanding which signaling pathways contribute to antipsychotic efficacy and side effects, for instance, will in turn enable the design of better antipsychotic drug candidates and, ultimately, lead to safer and more effective therapies for patients. However, only a small number of functionally selective GPCR ligands have been reported to date (5–9). In addition to the paucity of such

ligands, very little purposeful attention has been devoted to creating and annotating ligands with distinct patterns of functional selectivity. In particular, although β -arrestin–biased ligands of G_q and G_s-coupled GPCRs are known (9, 10), β -arrestin–biased GPCR ligands that selectively activate β -arrestin signaling pathways over G_i-coupled pathways have not been reported.

Aripiprazole (OPC-14597, 1), an FDA-approved atypical antipsychotic drug, was one of the first functionally selective D₂ receptor (D₂R or D₂) ligands identified (7, 11, 12). Although aripiprazole was initially described as a partial D₂R agonist, on the basis of assays performed in whole animals and isolated tissues (13–15), it was later demonstrated that aripiprazole could behave as a full agonist, a partial agonist, or an antagonist at D₂R depending upon the signaling readout and cell type interrogated (7, 11, 16, 17). However, structure–functional–selectivity relationships (SFSR) of the aripiprazole scaffold have not been studied and only modest structure activity relationships (SAR) have been reported (15, 18). In this paper, we report our discovery that UNC9975 (2), UNC0006 (3), and UNC9994 (4), analogs of aripiprazole, represent unprecedented β -arrestin–biased D₂R ligands; these compounds are also unique β -arrestin–biased ligands for G_i-coupled GPCRs. Here, we describe the design, synthesis, and in vitro and in vivo activities of these unique β -arrestin–biased chemical probes. We demonstrate, using wild-type and β -arrestin-2 knockout mice, that the atypical antipsychotic-like profile of these β -arrestin–biased D₂R agonists requires β -arrestin-2. The development of these unique, functionally selective, β -arrestin–biased D₂R ligands provides the biomedical community with valuable chemical tools for probing signaling pathways essential for antipsychotic efficacy and side effects.

Results

Discovery of β -Arrestin–Biased Dopamine D₂ Ligands UNC9975, UNC0006, and UNC9994. Because aripiprazole (1) has been shown to be a functionally selective D₂R ligand (7, 11) and because the SFSRs of the aripiprazole scaffold have not been studied, we intensely explored four regions of the aripiprazole template to

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identify D₂R ligands with distinct functional selectivity patterns (Fig. 1). We investigated various substituents (mono- and disubstitution) on the left-hand side phenyl ring, several cyclic amino groups, and various linkers in the middle region, along with a variety of bicyclic aromatic moieties at the right-hand side of the scaffold. Our intention was to modulate both the conformational preferences and substituent binding effects while retaining the core pharmacophore. In total, we prepared >150 unique compounds using the synthetic procedures described in *SI Methods*. These compounds were subsequently evaluated in dopamine D₂ receptor binding, G_i-regulated cAMP production, and G protein-independent β -arrestin-2 translocation assays. Although most of these compounds (15 representative compounds are summarized in Table S1) strongly activated both G_i-regulated cAMP accumulation and G protein-independent β -arrestin-2 recruitment in a manner similar to aripiprazole, we discovered UNC9975 (2), UNC0006 (3), and UNC9994 (4) as unprecedented β -arrestin-biased D₂R ligands, which were simultaneously partial agonists of β -arrestin-2 translocation and antagonists of G_i-regulated cAMP production (see below).

UNC9975, UNC0006, and UNC9994 Do Not Activate D₂-Mediated G_i-Regulated Inhibition of cAMP Production. UNC9975, UNC0006, and UNC9994 were initially evaluated in the D₂-mediated cAMP accumulation assay, which measures inhibition of isoproterenol-stimulated cAMP production via the G_i-coupled signaling pathway (19). UNC9975, UNC0006, and UNC9994 did not activate this G_i-mediated signaling pathway, in stark contrast to aripiprazole, which was a potent partial agonist (EC_{50} = 38 nM, E_{max} = 51%) (Fig. 2A). As expected, quinpirole (20) was a potent full agonist (Fig. 2A) whereas common typical and atypical antipsychotic drugs were antagonists in this assay. When tested for agonist activity at D₂ receptors to decrease basal cAMP in the absence of isoproterenol, none of the compounds exhibited activity. In addition, HEK293T cells devoid of D₂ receptors were assayed in parallel and displayed no inhibition of isoproterenol-stimulated cAMP, either by aripiprazole or by UNC9975 (Fig. S1), demonstrating that the effect observed in D₂ receptor-expressing cells is solely due to signaling through the D₂ receptors.

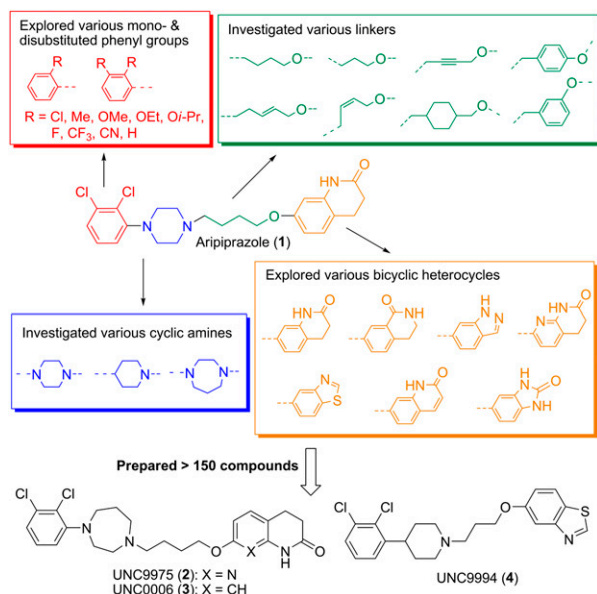


Fig. 1. Discovery of unprecedented β -arrestin-biased D₂R ligands UNC9975, UNC0006, and UNC9994 via exploring multiple regions of the aripiprazole scaffold.

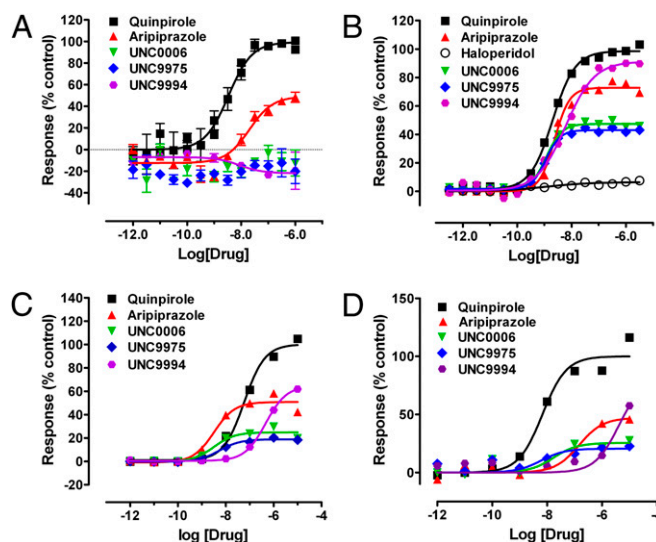


Fig. 2. UNC9975, UNC0006, and UNC9994 are functionally selective, β -arrestin-biased dopamine D₂ partial agonists. (A) Activity of UNC9975, UNC0006, UNC9994, aripiprazole, and quinpirole in the D₂-mediated G_i-coupled isoproterenol-stimulated cAMP production assay using HEK293T cells expressing the dopamine D₂ receptor and GloSensor-22F. UNC9975, UNC0006, and UNC9994 did not activate this G_i-mediated signaling pathway whereas aripiprazole (EC_{50} = 38 nM, pEC_{50} = 7.4 ± 0.1 , E_{max} = $51 \pm 5\%$) was a partial agonist and quinpirole (EC_{50} = 3.2 nM, pEC_{50} = 8.49 ± 0.07 , E_{max} = $100 \pm 3\%$) was a full agonist. Data are representative of at least two independent experiments. (B) Activity of UNC9975, UNC0006, UNC9994, aripiprazole, quinpirole, and haloperidol in the D₂-mediated β -arrestin-2 translocation Tango assay using HTLA cells transfected with a D₂V₂-TCS-tTA construct. UNC9975 (EC_{50} = 1.1 nM, pEC_{50} = 8.95 ± 0.03 , E_{max} = $43 \pm 0.5\%$), UNC0006 (EC_{50} = 1.2 nM, pEC_{50} = 8.91 ± 0.03 , E_{max} = $47 \pm 1\%$), UNC9994 (EC_{50} = 6.1 nM, pEC_{50} = 8.22 ± 0.09 , E_{max} = $91 \pm 3\%$), and aripiprazole (EC_{50} = 2.4 nM, pEC_{50} = 8.62 ± 0.03 , E_{max} = $73 \pm 1\%$) were partial agonists whereas haloperidol (antagonist control) had no agonist activity. Quinpirole (EC_{50} = 2.0 nM, pEC_{50} = 8.70 ± 0.05 , E_{max} = $100 \pm 2\%$) was used as a positive control. Data are representative of at least two independent experiments. (C) Activity of UNC9975, UNC0006, UNC9994, aripiprazole, and quinpirole in the D₂-mediated β -arrestin-2 translocation DiscoveRx assay with 20 h stimulation. UNC9975 (EC_{50} = 5.7 nM, pEC_{50} = 8.24 ± 0.20 , E_{max} = $19 \pm 1\%$), UNC0006 (EC_{50} = 3.2 nM, pEC_{50} = 8.49 ± 0.15 , E_{max} = $25 \pm 1\%$), UNC9994 (EC_{50} = 448 nM, pEC_{50} = 6.35 ± 0.07 , E_{max} = $64 \pm 2\%$), and aripiprazole (EC_{50} = 56 nM, pEC_{50} = 7.25 ± 0.04 , E_{max} = $100 \pm 2\%$) was used as a positive control. Data are representative of at least two independent experiments. (D) Activity of UNC9975, UNC0006, UNC9994, aripiprazole, and quinpirole in the D₂-mediated BRET-based β -arrestin-2 recruitment assay using HEK293 cells expressing GRK2. UNC9975 (EC_{50} = 6.0 nM, pEC_{50} = 8.22 ± 0.49 , E_{max} = $20 \pm 3\%$), UNC0006 (EC_{50} = 17 nM, pEC_{50} = 7.77 ± 0.38 , E_{max} = $25 \pm 4\%$), UNC9994 (EC_{50} > 1,000 nM, E_{max} > 50%), and aripiprazole (EC_{50} = 145 nM, pEC_{50} = 6.84 ± 0.18 , E_{max} = $47 \pm 4\%$) were all partial agonists that promote β -arrestin recruitment to D₂ receptors. Quinpirole (EC_{50} = 6.7 nM, pEC_{50} = 8.17 ± 0.15 , E_{max} = $100 \pm 5\%$) was used as a positive control. Data are representative of at least three independent experiments.

UNC9975, UNC0006, and UNC9994 Induce D₂-Mediated β -Arrestin-2 Translocation as Partial Agonists. To assess the effects of these compounds on recruiting β -arrestin-2 to D₂ receptors, we used a D₂-mediated β -arrestin-2 translocation Tango assay that is highly sensitive to β -arrestin-2 recruitment (21). In this assay, UNC9975, UNC0006, and UNC9994 were potent (EC_{50} < 10 nM) partial agonists for β -arrestin-2 recruitment to D₂ receptors, similar to aripiprazole (Fig. 2B). Whereas UNC9975 (E_{max} = 43%) and UNC0006 (E_{max} = 47%) were less efficacious than aripiprazole (E_{max} = 73%), UNC9994 (E_{max} = 91%) was more efficacious than aripiprazole and approached the activity of the full agonist quinpirole. Haloperidol (22), a typical antipsychotic (along with all other typical and atypical antipsychotic drugs

tested) did not activate D₂-mediated β -arrestin-2 translocation. Because HTLA cells (a HEK293-derived cell line stably expressing a tTA-dependent luciferase reporter gene and a β -arrestin-2-TEV fusion protein) transfected with a D₂V₂-TCS-tTA construct (21) were used in this assay, we tested these compounds in parallel in HTLA cells transfected with a V₂-TCS-tTA construct (21) to investigate the possibility that the observed effect was due to compound acting via the V₂ tail or some other nonreceptor-mediated arrestin pathway. As expected, UNC9975, UNC0006, aripiprazole, and quinpirole (a positive control for D₂) were inactive in V₂ receptor-expressing cells whereas arginine vasopressin (a positive control for V₂) was a full agonist (Fig. S24). These results demonstrate that the effect of UNC9975, UNC0006, UNC9994, and aripiprazole observed in D₂V₂ receptor-expressing cells is due to the compounds acting via the D₂ receptor, not at the V₂ tail.

To confirm this finding from the Tango assay, which has downstream amplification of signaling, we tested these compounds in an orthologous assay: the D₂-mediated β -arrestin-2 recruitment DiscoverX approach, which uses a β -galactosidase fragment complementation-based technology to monitor the interaction of β -arrestin with GPCRs (23). As shown in Fig. 2C and Fig. S2B, UNC9975 (EC₅₀ = 5.7 nM), UNC0006 (EC₅₀ = 3.2 nM), and aripiprazole (EC₅₀ = 3.4 nM) potently activated D₂-mediated β -arrestin-2 translocation. Although UNC9994 (EC₅₀ = 448 nM) was less potent, this compound (E_{\max} = 64%) had a higher efficacy than aripiprazole (E_{\max} = 51%), UNC9975 (E_{\max} = 19%), and UNC0006 (E_{\max} = 25%) (Fig. 2C). Quinpirole (EC₅₀ = 56 nM, E_{\max} = 100%) was used as a positive control in this assay. These findings are consistent with the results obtained in the D₂ β -arrestin-2 Tango assay.

Aripiprazole has been previously reported to be either an antagonist (17) or a partial agonist (24) for β -arrestin-2 recruitment using the bioluminescence resonance energy transfer (BRET)-based assay. Therefore, we also assessed the effects of UNC9975, UNC0006, and UNC9994 on D₂-mediated β -arrestin-2 recruitment using this BRET-based approach (17). In HEK293T cells wherein G protein-coupled receptor kinase 2 (GRK2) along with the D₂-dopamine receptor was expressed, aripiprazole, UNC9975, UNC0006, UNC9994, and aripiprazole all displayed partial agonist activity for D₂-mediated β -arrestin-2 recruitment (Fig. 2D). Although UNC9994 was less potent than aripiprazole, UNC9975, and UNC0006, UNC9994 had a higher efficacy than aripiprazole, UNC9975, and UNC0006 (Fig. 2D). Notably, compound activity to recruit β -arrestin-2 was sensitive to the expression of GRK2 in this BRET-based assay, as all compounds required GRK2 coexpression to induce detectable BRET signals in response to β -arrestin-2 recruitment (Fig. 2D and Fig. S2C). Therefore, using three orthogonal assay platforms, we confirmed that UNC9975 and UNC0006 were potent partial agonists for β -arrestin-2 recruitment with slightly lower efficacy than aripiprazole whereas UNC9994 was a higher efficacy and lower potency partial agonist for β -arrestin-2 translocation compared with aripiprazole.

We next examined the effects of UNC9975 and UNC0006 on inducing D₂ receptor endocytosis (internalization) by assessing the cell surface fluorescence of D₂ receptors using flow cytometry (25) in HEK293 cells coexpressing GRK2 and β -arrestin-2. D₂ receptor internalization in response to treatment with UNC9975, UNC0006, and aripiprazole (1 μ M for 1 h) did not reach statistical significance (Fig. S3). No internalization was observed in cells treated with haloperidol (as a negative control). As expected, quinpirole (a positive control) significantly increased receptor internalization.

To assess the effects of UNC9975 and UNC0006 on β -arrestin-mediated signaling, we evaluated these two compounds in the extracellular signal-regulated kinase (ERK) phosphorylation (p-ERK) reporter assay, which is capable of detecting extended β -arrestin-mediated ERK phosphorylation [following a relatively long incubation (4 h)] (10). In the HEK293T cells transfected with D₂ receptors, UNC9975 (EC₅₀ = 2.2 nM, E_{\max} = 32%) and

UNC0006 (EC₅₀ = 3.2 nM, E_{\max} = 33%) were potent partial agonists (Fig. S24). The potencies and efficacies of UNC9975 and UNC0006 are similar to those of aripiprazole (EC₅₀ = 1.8 nM, E_{\max} = 39%) in this assay. Importantly, coexpression of β -arrestin-2 and GRK2 significantly enhanced the efficacies of UNC9975, UNC0006, and aripiprazole in this signaling assay (Fig. S4B). In addition, UNC9975 (E_{\max} = 13%) and UNC0006 (E_{\max} = 11%) were inactive whereas aripiprazole was a potent partial agonist (EC₅₀ = 6.3 nM, E_{\max} = 49%) in the D₂ dopamine receptor-mediated p-ERK immunofluorescence assay (Fig. S4C), which measures the rapid phase of G protein-mediated ERK phosphorylation (5 min incubation) (26). These results are consistent with the cAMP biosensor assay results (see above) and further indicate that UNC9975 and UNC0006 lack appreciable agonism via canonical G_i-dependent pathways.

Taken together, these multiple functional activity profiling studies demonstrate that UNC9975, UNC0006, and UNC9994 are β -arrestin-biased D₂R ligands that selectively activate β -arrestin recruitment and signaling and are simultaneously *inactive* at G_i-mediated signal transduction pathways.

Selectivity of UNC9975, UNC0006, and UNC9994 and PK Parameters of UNC9975. Consistent with their potencies in D₂ functional assays, UNC9975 and UNC0006 displayed high affinities (K_i < 10 nM) similar to aripiprazole in the D₂ antagonist radioligand competition binding assay whereas UNC9994 displayed a lower binding affinity (K_i = 79 nM) to D₂R than UNC9975, UNC0006, and aripiprazole (Table 1). Although UNC9975, UNC0006, and UNC9994 also had high affinity for the D₃-dopamine receptor, they displayed low affinities for other dopamine receptors (i.e., D₁, D₄, and D₅; Table 1). At serotonin [as known as 5-hydroxytryptamine (5-HT or 5HT)] receptors, UNC9975, UNC0006, and UNC9994 displayed moderate to high binding affinities (K_i = 0.6–500 nM) for 5HT_{2A}, 5HT_{2B}, 5HT_{2C}, and 5HT_{1A}, but were significantly less potent in functional assays [Ca^{2+} mobilization fluorometric imaging plate reader (FLIPR) or cAMP biosensor]; UNC9975, UNC0006, and UNC9994 were antagonists at 5HT_{2A} and 5HT_{2B} and agonists at 5HT_{2C} and 5HT_{1A}. In addition, UNC9975, UNC0006, and UNC9994 had relatively high affinities to H₁-histamine receptor (K_i < 10 nM) but were less potent antagonists in H₁ functional assays. In general, UNC9975 and UNC0006 displayed a similar GPCR selectivity profile to aripiprazole. UNC9994 was less potent against most of these GPCRs compared with UNC9975, UNC0006, and aripiprazole.

Table 1. Radioligand binding and functional activities of UNC9975, UNC0006, and UNC9994 at select GPCRs

Receptor/assay	Binding affinity or potency, nM*			
	Aripiprazole	UNC9975	UNC0006	UNC9994
D ₂ /binding (K_i)	8.0	2.6	5.0	79
D ₁ /binding (K_i)	895	1,040	825	4,000
D ₃ /binding (K_i)	19	11	16	17
D ₄ /binding (K_i)	251	178	200	138
D ₅ /binding (K_i)	1,051	513	615	>10,000
5HT _{2A} /binding (K_i)	40	7.4	16	140
5HT _{2A} /FLIPR (pA ₂)	417	102	331	6,600
5HT _{2B} /binding (K_i)	1.4	1.1	0.6	25
5HT _{2B} /FLIPR (IC ₅₀)	98	76	115	501
5HT _{2C} /binding (K_i)	250	99	115	512
5HT _{2C} /FLIPR (EC ₅₀)	1,900	324	363	1,550
5HT _{1A} /binding (K_i)	18	29	60	26
5HT _{1A} /cAMP (EC ₅₀)	450	1,800	2,500	933
H ₁ /binding (K_i)	6.0	6.1	4.5	2.4
H ₁ /FLIPR (pA ₂)	52	35	22	79

* K_i , IC₅₀, pA₂, or EC₅₀ values are the average of at least two duplicate experiments with SD values that are threefold less than the average.

In mouse pharmacokinetic (PK) studies, both UNC9975 and aripiprazole displayed high exposure levels in brain and excellent CNS penetration (Table S2). Although the brain exposure level of UNC9975 was about threefold lower, UNC9975 had a longer half-life in brain and a higher brain/plasma ratio over 24 h compared with aripiprazole. The excellent in vivo PK parameters of UNC9975 make it a suitable tool for in vivo pharmacodynamic studies.

UNC9975 and UNC9994 Exhibit Antipsychotic Activity in Vivo That Is Attenuated in β -Arrestin-2 Knockout Mice. To correlate the in vitro functional selectivity profiles with potential in vivo therapeutic effects, we evaluated the inhibition of psychostimulant or psychotomimetic-induced hyperlocomotion in mice by UNC9975, both of which are well-established pharmacological mouse models for assessing potential antipsychotic activity (27, 28). We first evaluated the ability of UNC9975 to inhibit D-amphetamine-induced hyperlocomotion as detailed previously (27) in inbred C57BL/6 mice. As shown in Fig. 3*A*, i.p. administration of UNC9975 dose dependently inhibited D-amphetamine-induced hyperlocomotion. UNC9975 exhibited an efficacy and potency similar to aripiprazole ($ED_{50} = 0.38$ mg/kg vs. 0.36 mg/kg) in this in vivo model (Fig. 3*B*).

We next evaluated the effects of UNC9975 on psychotomimetic-induced hyperlocomotion in response to the NMDA receptor antagonist phencyclidine (PCP) (28). Because β -arrestin-2 knockout mice have a reduced locomotor response to amphetamine (3), we used PCP to induce locomotion in these animals. To assess the potential involvement of β -arrestin in the effects of UNC9975, β -arrestin-2 knockout mice and wild-type littermate controls (29) were administered increasing doses of UNC9975 followed by PCP administration. UNC9975 potently and dose dependently inhibited PCP-induced hyperlocomotion in wild-type mice (Fig. 3*C*); however, this antipsychotic-like activity of UNC9975 was significantly attenuated in β -arrestin-2 knockout mice (Fig. 3*D*). Significantly, the in vivo potency of UNC9975 was greatly decreased in β -arrestin-2 knockout mice (β -arrestin-2 knockout $ED_{50} = 0.75$ mg/kg vs. wild-type $ED_{50} = 0.26$ mg/kg) (Fig. 3*E*). In striking contrast, aripiprazole displayed no significant differences in potency or efficacy in wild-type ($ED_{50} = 0.13$ mg/kg) and β -arrestin-2 knockout mice ($ED_{50} = 0.15$ mg/kg) (Fig. 3*F*).

To confirm these findings, we next evaluated the effects of UNC9994 on PCP-induced hyperlocomotion in wild-type and β -arrestin-2 knockout mice. UNC9994 (2 mg/kg, i.p.) markedly inhibited PCP-induced hyperlocomotion in wild-type mice (Fig. 4*A*). Importantly, this significant antipsychotic-like activity of UNC9994 was completely abolished in β -arrestin-2 knockout mice (Fig. 4*B*).

Because UNC9975 has 5HT_{2A} antagonist activity (Table 1) and 5HT_{2A} antagonism can be a significant contributor to the efficacy of antipsychotic drugs (30, 31), we evaluated SR46349B (a potent and selective 5HT_{2A} antagonist) (32) and clozapine (a classic atypical antipsychotic with potent antagonist activity at 5HT_{2A} and other aminergic GPCRs) (33) in the PCP-induced hyperlocomotion model in wild-type and β -arrestin-2 KO mice. We found that SR46349B and clozapine were equally effective at suppressing PCP-induced locomotion in β -arrestin-2 KO mice vs. wild-type mice (Fig. S5), indicating that antagonism of 5HT_{2A} receptors is similar in both genotypes. These observations suggest that the attenuation in antipsychotic drug-like activity of UNC9975 in β -arrestin-2 KO mice is likely not due to activity at 5-HT_{2A} receptors.

Taken together, our results strongly suggest that the antipsychotic drug-like activity of UNC9975 and UNC9994 requires β -arrestin and this is likely mediated through the dopamine D₂ receptors in mice.

UNC9975 and UNC0006 Induce Catalepsy in β -Arrestin-2 Knockout Mice but Not in Wild-Type Mice. To compare potential extrapyramidal side effects of UNC9975, UNC0006, and aripiprazole, we evaluated these compounds along with haloperidol (as a positive control) in a standard drug-induced catalepsy model (34), using wild-type and β -arrestin-2 knockout mice. As shown in

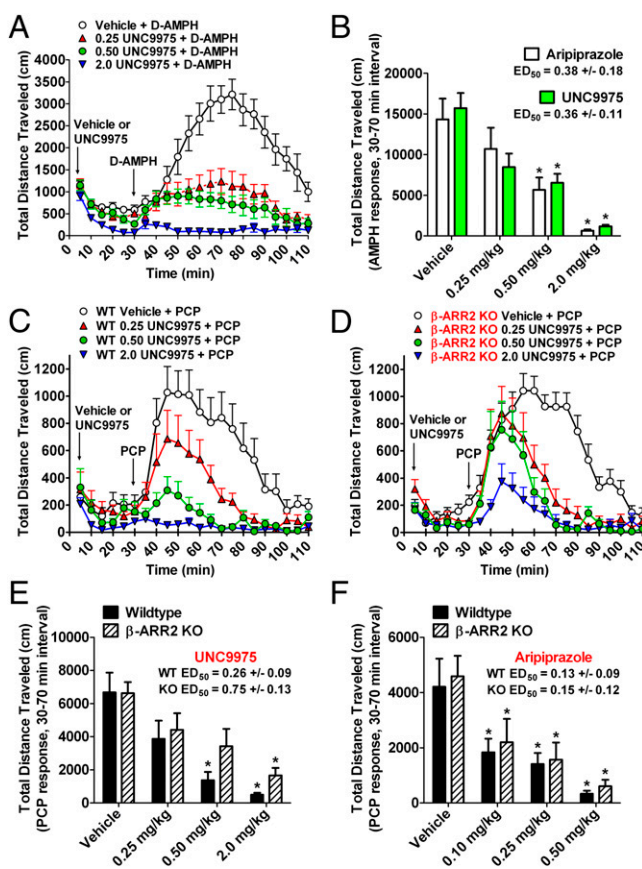


Fig. 3. UNC9975 exhibits potent antipsychotic-like activity in mouse hyperlocomotion studies that is attenuated in β -arrestin-2 knockout mice. (A) Locomotor responses in inbred C57BL/6 mice are shown as 5-min binned intervals to vehicle or different doses (i.p.) of UNC9975 followed 30 min later by 3 mg/kg D-amphetamine (AMPH, i.p.). (B) Bar graph of distance traveled after AMPH administration (30- to 70-min time interval). C57BL/6 mice were given vehicle or different doses of UNC9975 or aripiprazole 30 min before AMPH treatment. $n = 8$ animals/group. * $P < 0.05$, vs. vehicle + 3 mg/kg AMPH group. (C and D) Locomotor activities shown as 5-min binned intervals of wild-type (WT) or β -arrestin-2 knockout (β -ARR2 KO) littermate mice given vehicle or different doses of UNC9975 followed 30 min later with 6 mg/kg phencyclidine (PCP, i.p.). (E) Bar graph of distance traveled by WT and β -ARR2 KO mice after PCP administration (30- to 70-min time interval) as shown in C and D. $n = 14$ WT and β -ARR2 KO pairs/group. * $P < 0.05$, vs. vehicle + 6 mg/kg PCP group. (F) Bar graph of distance traveled by WT and β -ARR2 KO mice after aripiprazole injection followed by PCP treatment (30- to 70-min time interval). $n = 8$ littermate WT and β -ARR2 KO pairs/group. * $P < 0.05$, vs. vehicle + 6 mg/kg PCP group.

Fig. 5*A* and *B*, UNC9975, UNC0006, or aripiprazole (5.0 mg/kg) failed to significantly induce catalepsy in wild-type mice at either 30 or 60 min after treatment, whereas haloperidol (2.0 mg/kg) induced significant catalepsy at both time points. In notable contrast to the results in wild-type animals, the β -arrestin-biased dopamine D₂ ligands UNC9975 and UNC0006 significantly induced catalepsy in β -arrestin-2 knockout mice 60 min after treatment (Fig. 5*B*). By comparison, aripiprazole, which activates both G_i-coupled and β -arrestin-mediated pathways, did not induce catalepsy in β -arrestin-2 knockout mice or in wild-type mice (Fig. 5*B*). Collectively, these results suggest that β -arrestin recruitment and signaling are protective against motoric side effects.

Discussion

Through a combined medicinal chemistry and comprehensive in vitro and in vivo pharmacological profiling approach, we designed, synthesized, and characterized unique D₂ β -arrestin-

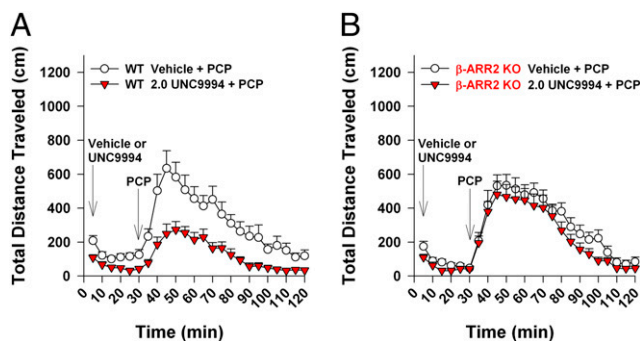


Fig. 4. UNC9994 exhibits potent antipsychotic-like activity in mouse hyperlocomotion studies that is completely abolished in β -arrestin-2 knockout mice. (A and B) Locomotor activities shown as 5-min binned intervals for wild-type (WT) or β -arrestin-2 knockout (β -ARR2 KO) littermate mice given vehicle or 2.0 mg/kg UNC9994 (i.p.) followed 30 min later with 6 mg/kg phencyclidine (PCP, i.p.). $n = 10$ –13 WT and β -ARR2 KO pairs/group.

biased agonists and demonstrated their atypical antipsychotic drug-like activities in vivo. These chemical probes represent unprecedented functionally selective β -arrestin-biased dopamine D_2 R ligands that exhibit antipsychotic activity in vivo. This study thus represents a successful proof-of-concept for how functionally selective GPCR ligands can be discovered and validated. Additionally, our findings show that on the basis of the β -arrestin bias of these compounds, β -arrestin emerges as an important contributor to both antipsychotic drug efficacy and antipsychotic side effects.

Our robust diversity-oriented synthetic approach capitalized on the aripiprazole scaffold to generate, initially, hundreds of analogs. Among them, UNC9975, UNC0006, and UNC9994 were discovered as unique, β -arrestin-biased functionally selective D_2 ligands. These compounds are partial agonists that induce D_2 receptor-mediated β -arrestin recruitment and signaling and simultaneously are inactive at G_i -dependent signaling. Further, it should be acknowledged that whereas UNC9975, UNC0006, and UNC9994 promoted the recruitment of β -arrestin to D_2 receptors, these studies were performed in heterologous cells expressing β -arrestin-2 and GRK2. Whether these compounds stimulate β -arrestin recruitment and signaling in native D_2 receptor-expressing neurons is the subject of ongoing investigations. Interestingly, although aripiprazole, UNC0006, and UNC9975 are all agonists for β -arrestin recruitment and signaling, in contrast to quinpirole, none of these ligands promoted robust internalization of D_2 receptors, a trafficking phenomenon commonly involving β -arrestins (35). This inability of aripiprazole to induce significant receptor internalization is consistent with previous findings (7),

suggesting that a common property of aripiprazole-based ligands may be the inability to promote robust D_2 receptor internalization. This lack of internalization may bode well for the eventual translation of such β -arrestin-biased ligands to the clinic as drugs that induce internalization would ultimately induce tachyphylaxis and receptor down-regulation (36).

More significantly, the in vivo activity of UNC9975 indicated a strict requirement for β -arrestin-2 for both full antipsychotic activity and protection against motoric side effects. Similar to aripiprazole, this β -arrestin-biased ligand shows a potent ability to suppress both D-amphetamine and phencyclidine-induced hyperlocomotion in mice, indicating that the compound possesses antipsychotic drug-like activities in vivo. Significantly, the antipsychotic drug-like activities of UNC9975 were attenuated in β -arrestin-2 knockout mice, indicating that β -arrestin-2 is required in vivo for full activity. Interestingly, previous studies have determined that the locomotor responses induced by the non-selective direct (apomorphine) and indirect (amphetamine) agonists are attenuated in β -arrestin-2 knockout mice (3). Importantly, the mechanisms by which β -arrestin influences these locomotor responses in vivo and to what extent this β -arrestin-2 knockout effect is specific to D_2 receptors are currently unknown. Here we observe that in β -arrestin-2 knockout mice the unique β -arrestin-biased ligands UNC9975 and UNC9994, but not the other atypical drugs including aripiprazole, clozapine, and SR46349B, show an attenuated ability to inhibit PCP-induced locomotion. These findings demonstrate that β -arrestin-2 is required for the full antipsychotic drug-like activity of UNC9975 and UNC9994. However, further mechanistic studies will be required to determine how D_2 -mediated β -arrestin recruitment and signaling by UNC9975 and UNC9994 are essential for these compounds' antipsychotic activity in vivo.

With the exception of aripiprazole, all Food and Drug Administration-approved typical and atypical antipsychotic medications (e.g., haloperidol, chlorpromazine, clozapine, and risperidone) share the common property of antagonizing D_2 -mediated G protein-dependent and -independent signaling (17). Indeed, typical and atypical antipsychotic drugs are antagonists at G_i -mediated signaling and arrestin-ergic pathways. It is currently unknown what, if any activity, any of these compounds have at potential G_o -mediated signaling. This antagonism at both signaling pathways is thought to underlie the therapeutic benefit to prevent psychotic symptoms but can also cause serious extrapyramidal side effects including catalepsy and other motor dyskinesias (37). Although aripiprazole, UNC0006, and UNC9975 do not induce catalepsy in wild-type mice at doses at which antipsychotic drug-like therapeutic activities are measured, UNC0006 and UNC9975 resemble haloperidol in inducing catalepsy in β -arrestin-2 knockout mice. This propensity to cause catalepsy in the absence of β -arrestin-2 suggests that UNC0006 and UNC9975 signal through β -arrestin-2 in vivo and that this signaling may protect against motoric side effects due to antagonism at D_2 R-mediated G_i pathways.

These findings have obvious implications for the development of therapeutic approaches for treating schizophrenia and related disorders. Atypical antipsychotic drugs, which differ from older medications (e.g., haloperidol and chlorpromazine) by virtue of their reduced propensity to induce motoric side effects, are among the most widely prescribed medications. Many pharmacologic strategies that aim to target a multiplicity of non- D_2 receptor molecular targets (e.g., 5-HT_{2A} inverse agonists, 5-HT_{2C} agonists, mGluR_{2/3} agonists, NK-3 antagonists, sigma antagonists, D₁- or D₄-selective antagonists, and so forth) have led to a growing number of unsuccessful attempts to create safer and more effective atypical antipsychotic drugs (37–39). Our results suggest that atypical antipsychotic drugs with a unique mechanism of action may arise from β -arrestin-biased D_2 ligands. Although we do not know whether such compounds will have therapeutic benefits over existing medications, it is tempting to speculate that they may demonstrate special efficacies in psychotic disorders with a mood dysfunction given the prominent role of arrestin-ergic signaling in the action of lithium and related compounds (40, 41).

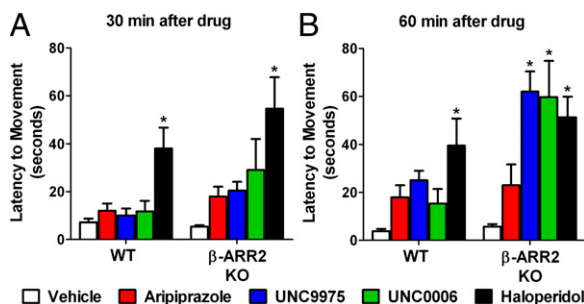


Fig. 5. UNC9975 and UNC0006 induce catalepsy in β -arrestin-2 knockout mice but not in wild-type littermates. (A and B) Wild-type (WT) and β -arrestin-2 knockout (β -ARR2 KO) littermate mice were administered (i.p.) vehicle, 5.0 mg/kg UNC9975, 5.0 mg/kg UNC0006, 5.0 mg/kg aripiprazole, or 2.0 mg/kg haloperidol. Catalepsy was assessed 30 and 60 min after drug injection using the inclined screen test where latency to move was scored. $n = 8$ WT and β -ARR2 KO pairs/group. * $P < 0.05$, vs. vehicle controls.

Methods

A complete description of chemical synthesis for all compounds and protocols for all biological experiments is detailed in *SI Methods*. ^1H and ^{13}C NMR spectra of UNC9975 (Fig. S6), UNC0006 (Fig. S7), and UNC9994 (Fig. S8) are provided in *SI Methods*.

D₂ β -Arrestin Recruitment Assay. Recruitment of β -arrestin to agonist-stimulated D_{2L} receptors was performed using a previously described “Tango”-type assay (21). Briefly, HTLA cells stably expressing β -arrestin-TEV protease and a tetracycline transactivator-driven luciferase were plated into 15-cm dishes in DMEM containing 10% FBS. Cells were transfected (calcium phosphate) with 20 μg of a D_{2V2}-TCS-tTA construct (21). The next day, cells were plated in white, clear-bottom, 384-well plates (Greiner; 10,000 cells/well, 50 μL /well) in DMEM containing 1% dialyzed FBS. The following day, the cells were challenged with 10 μL /well of reference agonist (6 μM) or D₂ test ligand (6 μM) \pm reference agonist prepared in HBSS, 20 mM Hepes (pH 7.4), and 18% DMSO (final ligand concentrations are 1 μM , final DMSO concentration is 3%). After 18 h, the medium was removed and replaced with 1 \times BriteGlo reagent (Promega), and luminescence per well was read using a TriLux plate reader (1 s/well). Data were normalized to vehicle (0%) and quinpirole (100%) controls and regressed using the sigmoidal dose-response function built into GraphPad Prism 4.0.

In Vivo Studies in Mice. All experiments were approved by the Institutional Animal Care and Use Committees at the University of North Carolina, Chapel Hill and Duke University. C57BL/6J wild-type and β -arrestin-2 knockout mice were housed under standard conditions: 12-h light/dark cycle with food and water provided ad libitum. Adult, age-matched male and female wild-type and β -arrestin-2 knockout drug-naïve mice were used for all behavioral testing.

Locomotor activity was assessed under standardized environmental conditions in 21 \times 21-cm Plexiglas chambers with photobeams spaced at 2.5 cm (AccuScan Instruments) as previously described (42). Mice were injected (i.p.) with vehicle (0.9% saline/0.2% acetic acid), aripiprazole (0.1, 0.25, 0.50, or 2.0 mg/kg), or UNC9975 (0.25, 0.50, or 2.0 mg/kg) and placed into the open field. For the studies with SR46349B and clozapine, mice were injected (i.p.) with vehicle (0.9% saline/50 mM tartaric acid), SR46349B (1.0 mg/kg), or clozapine (1.0 mg/kg) and placed into the open field. Thirty minutes later *D*-amphetamine (3 mg/kg) or phencyclidine (6.0 mg/kg) was administered and mice were immediately returned to the open field. Activity was monitored throughout this entire period. Horizontal activity was measured as the total distance traveled in centimeters. The means \pm SEMs of the locomotor responses were analyzed using Graphpad Prism 5.0. To estimate the half-maximal inhibitory concentration (ED₅₀), dose responses of total locomotor activity during the 90-min period after *D*-amphetamine or phencyclidine administration were plotted and best-fit decay curves were determined using a nonlinear regression one-phase decay equation. Cumulative locomotor responses were analyzed using a one-way ANOVA followed by Newman–Keuls multiple comparison tests with Graphpad Prism 5.0. Statistical significance was set at $P < 0.05$.

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